



STY-9961

- 1 -

MEGAKARYOCYTE DIFFERENTIATION FACTOR

## BACKGROUND OF THE INVENTION

## 5           1.     Field of the Invention

The present invention relates to a megakaryocyte differentiation factor, gene coding for the factor and a process for production thereof. The megakaryocyte differentiation factor is useful as a hemopoietic stimulating factor for megakaryocyte-platelet lineage.

## 2.     Description of Related Art

It is well known that various hemopoietic factors inducing the growth and differentiation of blood cells are involved in a process from hemopoietic stem cells to mature blood cells.

Although the life time of platelet is as short as 9 to 10 days, concentration of platelets in the blood is maintained rather constant during the stationary state. Moreover, when the number of platelets is artificially reduced by one of various available method in an experimental animals, the number of the platelets recovers in the blood in a few days. From these facts, it is supposed that factors which stimulate formation of platelets are present, and so far a great effort has been made to identification of the factors.

It is considered that at least two regulatory factors are involved in the megakaryocyte-platelet hemopoietic lineage. The first factor by itself stimulates formation of megakaryocyte colonies and is called a megakaryocyte colony stimulating factor. The

- 2 -

second factor by itself does not have an activity to stimulate formation of megakaryocyte colonies, but in combination with the first factor, increases the number of megakaryocyte colonies and stimulates the  
5 differentiation thereof, and is called a megakaryocyte potentiator.

The former includes interleukin 3, and granulocyte/macrophage colony stimulating factor, and the latter includes erythropoietin, macrophage colony  
10 stimulating factor, interleukin 6, 7 and 11, LIF, and the like. Some of these factors actually exhibit in vivo effect of increasing the number of platelets or shortening the time required to recover the number of platelets (Hideaki Mizoguchi: Tanpakushitsu Kakusan Koso  
15 36, 1195, 1991 in Japanese).

However, most of these factors exhibit a diversity of biological activities other than participation in differentiation of blood cells in various hemopoietic lineages including differentiation in  
20 megakaryocyte-platelet lineages. For example, although IL-6 and IL-11 actually exhibit in vivo thrombopoietic action, they stimulate production of acute phase protein, and in severe cases, cause cachexia. Moreover, IL-6 is accompanied with various clinical problems; for example,  
25 it is possible for IL-6 to stimulate the growth of mesangium cells in the kidney resulting in renal failure (Tadashi Matsuda et al., Tanpakushitsu Kakusan Koso, 36, 1184, 1991 in Japanese). In addition, since IL-6 does not exhibit a high blood level during a thrombocytopenic  
30 phase, it is not considered as a physiological factor.

Platelets play an important role in a

- 3 -

hemostatic mechanism. Diseases involving decrease of platelets (Fanconi's syndrome, megakaryocytic thrombocytopenia, aplastic anemia, and the like) are clinically dangerous, and in particular hemorrhaging cannot be controlled. Therefore, it is considered that isolation and identification of a factor which stimulates production of platelets is useful to prevent the above-mentioned danger.

Currently, bone marrow transplantation is becoming a powerful therapeutic means for treating leukemia etc., and the ratio of successful cases is increasing through the use of cytokines such as erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF) etc. At present a problem in the bone marrow transplantation is a decrease in the number of platelets, and if a thrombopoietic factor is available, it is expected that the ratio of successful cases will increase and a period of hospitalization will be shortened. Not only hemopoietic diseases but also thrombocytopenia in chemotherapy and radio isotopic therapy of cancers may be controlled by thrombopoietin.

The present inventors, considering the various above-mentioned difficulties with known factors, carried out various research to find a factor which stimulates production of platelets and is effective for treatment of patients having thrombocytopenia or insufficient platelet function, and as a result, the present inventors found a novel factor which stimulates differentiation of megakaryocytes, cloned a gene coding for said factor, constructed an expression vector, and succeeded in expressing the gene to produce said factor.

- 4 -

## SUMMARY OF THE INVENTION

Accordingly, the present invention provides a megakaryocyte differentiation factor having the following properties;

- 5           (1) stimulating differentiation of megakaryocytes;
- (2) exhibiting a molecular weight of 55 to 57 kD as determined by gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and having no intermolecular disulfide linkage;
- 10           (3) exhibiting an isoelectric point of  $6.5 \pm 0.5$ ; and
- (4) having at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9 in the Sequence Listing.

The present invention also provides a gene coding for the megakaryocyte differentiation factor.

The present invention further provides an expression vector comprising the gene coding for the megakaryocyte differentiation factor.

The present invention moreover provides a host transformed with the expression vector.

The present invention still further provides a process for production of the megakaryocyte differentiation factor using said host.

## BRIEF EXPLANATION OF DRAWINGS

25           Figure 1 shows an elution profile of protein (A280 ●) and a megakaryocyte differentiation factor (acetylcholine esterase activity ○) from a Q-Sepharose column, wherein the elution was carried out by NaCl concentration gradient (0 to 1.0M) to obtain fractions 1 to 120.

30           Fig. 2 shows an elution profile of protein

- 5 -

(A280 ● ) and a megakaryocyte differentiation factor (acetylcholine esterase activity ○ ) from a Phenyl-Sepharose column, wherein the elution was carried out by ammonium sulfate concentration gradient (30 to 0%) and  
5 ethyleneglycol concentration gradient (0 to 50%) to obtain fractions Nos. 1 to 100, followed by 50% ethyleneglycol to obtain fraction Nos. 101 to 120.

Fig. 3 shows an elution profile of protein (A280 ● ) and a megakaryocyte differentiation factor (acetylcholine esterase activity ○ ) from an S-Sepharose  
10 column, wherein the elution was carried out by NaCl concentration gradient (0 to 0.5M) to obtain fractions Nos. 1 to 100.

Fig. 4 shows an elution profile of protein (A280 ) and megakaryocyte differentiation factor (acetylcholine esterase activity ○ ) from a Hiroad 26/60  
15 Superdex 75 Pg column, and a result of analysis of the resulting fractions by SDS-PAGE (lower part of the Figure).

20 Fig. 5 is an electrophoretic pattern showing a result of SDS-PAGE analysis of the purified megakaryocyte differentiation factor.

Fig. 6 shows a result of an isoelectric focusing of the purified megakaryocyte differentiation factor.

25 Fig. 7 is an electrophoretic pattern showing a result of sugar chain analysis for the purified megakaryocyte differentiation factor by SDS-PAGE, wherein the column 1 shows a result for a non-treated megakaryocyte differentiation factor, and the  
30 column 2 shows a result for an endoglycosidase F-treated megakaryocyte differentiation factor (note, the band near

- 6 -

the 35 kD position is derived from the enzyme preparation).

Fig. 8 is a graph comparing acetylcholine esterase activity of megakaryocytes derived from mouse bone marrow cells cultured for 5 days in the presence or absence of purified megakaryocyte differentiation factor (55 kDa protein) and with or without addition of IL-6.

Fig. 9 shows a result of acetylcholine esterase staining of mouse bone marrow cells cultured for 4 days after the addition of IL-3, in the presence (B) or absence (A) of purified megakaryocyte differentiation factor.

Fig. 10 shows a result of May-Gruenwald-Giemsa's staining of mouse bone marrow cells cultured for 4 days after the addition of IL-3, in the presence (B) or absence (A) of purified megakaryocyte differentiation factor.

Fig. 11 shows detection of RNA with a DNA probe (PCR product amplified with KY100 and N1065 described in Example 2.1) wherein the RNA was extracted from cultured Bombyx mori cells infected with recombinant virus (TP055-Bm NPV) comprising a gene coding for megakaryocyte differentiation factor (Lane B), cells infected with wild type virus (B6E) (Lane A) or from non-transformed cells (Lane C).

Fig. 12 is a graph showing an expression of megakaryocyte differentiation factor (TP55) in hemolymph of Bombyx mori after reparation by Matrex Blue A column chromatography.

DETAILED DESCRIPTION

As starting materials for isolating the present

- 7 -

megakaryocyte differentiation factor, human cells, such as human cancer cells, preferably human epidermoid carcinoma cells A431, particularly preferably human epidermoid carcinoma A431 grown in a protein-free medium  
5 can be mentioned.

In addition to the above-defined megakaryocyte differentiation factor, the present invention relates megakaryocyte differentiation factors obtainable from transformants such as cells or animals constructed by  
10 gene technology and having the same amino acid sequence as the above-defined megakaryocyte differentiation factor, an amino acid sequence wherein a portion of the above-defined megakaryocyte differentiation factor is deleted, an amino acid sequence wherein a portion of the  
15 above-defined megakaryocyte differentiation factor is replaced with other amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid sequence is added to the above-defined megakaryocyte differentiation factor, or having an amino acid sequence  
20 including a combination of said modifications.

Moreover, the present invention relates to megakaryocyte differentiation factor having the amino acid sequence shown in SEQ ID NO: 30, an amino acid sequence wherein a portion of the amino acid sequence  
25 shown in SEQ ID NO: 30 is deleted, an amino acid sequence wherein a portion of the amino acid sequence shown in SEQ ID NO: 30 is replaced with an other amino acid or amino acid sequence, or an amino acid sequence wherein one or more than one amino acid sequence is added to the  
30 amino acid sequence shown in SEQ ID NO: 30, or having an amino acid sequence including a combination of said

- 8 -

modifications.

The present invention also relates to genes coding for the above-mentioned megakaryocyte differentiation factors. The present invention further relates a process  
5 for production of the megakaryocyte differentiation factors using the gene by means of gene recombination technology. The gene recombination technology follows conventional procedures by using a synthetic or natural polynucleotide coding for the amino acid sequence of the  
10 native megakaryocyte differentiation factor, an amino acid sequence wherein a portion of the native amino acid sequence is deleted, an amino acid sequence wherein a portion of the native amino acid sequence is replaced with other amino acid or amino acid sequence, or an amino  
15 acid sequence wherein one or more than one amino acid is added to the native amino acid sequence, or coding for an amino acid sequence including a combination of said modifications, but not limited to the above.

The above-mentioned various modification can be  
20 carried out by a conventional technique such as site-specific a mutagenesis.

The number of amino acids involved in the modification such as addition, deletion or replacement is not limited, but as for addition the number of amino acid  
25 depends on the number of amino acids, for example, that of the functional peptide used in a hybrid protein with the megakaryocyte differentiation factor of the present invention or that of a signal peptide added to the present factor, namely depends on purpose of the  
30 modification, as for deletion the number of amino acids may be designed or determined so as to maintain



- 9 -

megakaryocyte differentiation activity and it is, for example, 1 to 30, preferably 1 to 20 or it can be that of region other than the active region of the present factor, and as for replacement the number of amino acids  
5 also may be designed or determined so as to maintain megakaryocyte differentiation activity and it is, for example, 1 to 10, preferably, 1 to 5.

An addition or improvement of an signal sequence, choice of host-vector system, and improvement of  
10 expression regulatory region may provide efficient expression. In addition, a host may be chosen to provide a glycosylated product. Moreover, a polynucleotide coding for at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9 may be used as a DNA probe for  
15 cloning a gene.

The present invention further provides a pharmaceutical composition comprising a megakaryocyte differentiation factor as an effective ingredient. The pharmaceutical composition is preferably used as a  
20 medicament for thrombocytopenia.

In addition, the present megakaryocyte differentiation factors can be used to obtain specific antibodies according to a known procedure.

Now, the present invention is explained in more  
25 detail.

(1) Starting material

As a starting material for obtaining a novel protein of the present invention, a culture supernatant of cells derived from human epidermoid carcinoma cell  
30 A431 (ATCC CRL 1555), rendered to be capable of growing in a protein-free medium according to Yamaguchi et al.

- 10 -

method (Yamaguchi N. et al., Cancer Res. 50, 7008, 1991), is mentioned. This cell line was designated as Human epidermoid carcinoma SBM 330, and deposited as FERM BP-3911 with the Fermentation Research Institute Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi Ibaraki-ken (307) Japan, on July 1, 1992.

(2) Assay method for megakaryocyte differentiation factor

To assay a megakaryocyte differentiation factor, megakaryocyte-series cell lines (for example, CMK cells or cells derived therefrom), or mouse bone marrow cells may be used. For example, activity of acetylcholine esterase which is known to be specifically detected in murine megakaryocytes is carried out using mouse bone marrow cells according to the Ishibashi et al. method (Ishibashi, T. et al., Proc. Natl. Acad. Sci. USA 86, 5953, 1989). In addition, histochemical detection of megakaryocytes is carried out by subjecting cultured bone marrow cells to acetylcholine esterase staining and May-Gruenwald-Gremsa's staining and the observing the shape of the stained cells.

(3) Purification of megakaryocyte differentiation factor

The megakaryocyte differentiation factor can be purified, for example, starting from a culture supernatant of A431 cells cultured in a protein-free medium, concentration by ultrafiltration, and column chromatography using, for example, Matrex Blue A (Amicon), Q-Sepharose (Pharmacia), phenyl-Sepharose (Pharmacia), S-Sepharose (Pharmacia) and Hiload 26/60

- 11 -

Superdex 75 (Pharmacia) alone or in combination. Protein is monitored by measuring A280 nm.

(4) Determination of partial amino acid sequence of megakaryocyte differentiation factor

5 To determine an amino acid sequence, the megakaryocyte differentiation factor purified in the section (3) is digested into fragments with a protease such as Achromobacter Protease I (API) at 37°C for 2 hours. The resulting peptide fragments are separated  
10 and recovered by reverse phase HPLC (acetonitrile gradient in 0.1% trifluoroacetic acid) using YMC-Pack AM-303 column. The peptide fragments thus obtained are subjected to a sequencer such as a gas-phase sequencer obtained from, for example, Applied Biosystem. A  
15 definite purification process and detailed properties of the megakaryocyte differentiation factor are described in Example 1.

The present invention also provides gene coding for megakaryocyte differentiation factor. The gene may be  
20 cDNA prepared from mRNA, genomic DNA, and synthetic DNA. For example, cDNA can be cloned by a polymerase chain reaction (PCR) using DNA (nucleotide) primers designed on the basis of partial amino acid sequences as shown in Example 1 of a megakaryocyte differentiation factor  
25 purified from the above-mentioned human cells, such as human epidermoid carcinoma cells, for example A431 cells. The cloning is described in detail in Examples 1 and 2.

The gene of the present invention further includes DNAs coding for protein or glycoprotein having  
30 megakaryocyte differentiation activity and hybridizing with the nucleotide sequence of SEQ ID NO: 30.

- 12 -

Nucleotide sequence of DNA cloned in Example 2 and an amino acid sequence predicted from the nucleotide sequence are shown in SEQ ID NO: 30.

Thus once an amino acid sequence is determined,  
5 various mutated megakaryocyte differentiation factors, such as a polypeptide, wherein one or more than one amino acid is added to the native amino acid sequence or the amino acid sequence shown in SEQ ID NO: 30 and maintaining megakaryocyte differentiation factor  
10 activity, a polypeptide wherein one or more than one amino acid is deleted from the native amino acid sequence or the amino acid sequence shown in SEQ ID NO: 30 and maintaining megakaryocyte differentiation factor activity, a polypeptide wherein one ore more than one  
15 amino acid is replaced with one or more than one other amino acid, in the native amino acid sequecne or the amino acid sequence shown in SEQ ID NO: 30, and maintaining megakaryocyte differentiation factor activity, or a polypeptide including a combination of the  
20 above-mentioned modifications such as addition, deletion and/or replacement of amino acids maintaining megakaryocyte differentiation factor activity can be designed and produced.

According to the present invention, although the  
25 nucleotide sequence shown in SEQ ID NO: 30 is disclosed, gene coding for the present megakaryocyte differentiation factor is not limited thereto. Once an amino acid sequence of the present native megakaryocyte differentiation factor or an amino acid sequence of a  
30 mutated megakaryocyte differentiation factor is determined, according to the degeneracy of genetic code,

- 13 -

various nucleotide sequences coding for the same amino acid sequence can be designed and prepared. In this case, codons used with a high frequency in a chosen host are preferably used.

5           A gene coding for the present megakaryocyte differentiation factor can be obtained as cDNA according to Example 2, but the gene is not limited to cDNA. Namely, once a nucleotide sequence coding for an amino acid sequence of native megakaryocyte differentiation  
10       factor is determined, a gene coding for the native megakaryocyte differentiation factor can be cloned as a cDNA according to a strategy different from the strategy definitely described herein, and moreover a desired gene can be cloned from the genome of a cell producing the  
15       megakaryocyte differentiation factor.

          Where a desired gene is cloned from the genome, various primer nucleotides or probe nucleotides used in Example 2 can be used as probes for screening genomic DNA fragments. Moreover, other probes designed on the basis  
20       of the nucleotide sequence described in SEQ ID NO: 30 can be used. General procedures for cloning a desired DNA from a genome are well known in the art (see Current Protocols In Molecular Biology, John Wiley & Sons, Chapters 5 and 6).

25           Gene coding for the native megakaryocyte differentiation factor of the present invention can also be prepared by chemical synthesis. It is easy in the art to chemically synthesize DNA using an automated DNA synthesizer, for example, Applied Biosystems 396 DNA/RNA  
30       synthesizer. Accordingly a person with ordinary skill in the art can easily synthesize DNA having the nucleotide

- 14 -

sequence shown in SEQ ID NO: 30.

A gene coding for the present native megakaryocyte differentiation factor using codons different from native codons, and a gene coding for a mutated megakaryocyte differentiation factor can be prepared by chemical synthesis as described above. Alternatively they can be obtained by site-specific mutagenesis using as a template a DNA or RNA having a nucleotide sequence shown in SEQ ID NO: 30 together with mutagenic primers (for example, see, Current Protocols In Molecular Biology, John Wiley & Sons, Chapter 8).

Once a gene coding for the present megakaryocyte differentiation factor is obtained, the gene can be used to produce a recombinant megakaryocyte differentiation factor according to a conventional gene recombination technology. Namely, a DNA coding for the present megakaryocyte differentiation factor is inserted into an appropriate expression vector, the vector is introduced to appropriate host cells, the transformed host cells are cultured, and the target megakaryocyte differentiation factor is recovered from the culture (cells or medium). The present megakaryocyte differentiation factor can be biochemically or chemically modified, for example, N-terminal acylated.

In addition, on the basis of the nucleotide sequence shown in SEQ ID NO: 30, a protein data base was searched with fasta Program (GCG package). As a result, the megakaryocyte differentiation factor belongs to the super family of serine protease inhibitor. On the other hand, in human leucocyte elastase inhibitor, chicken ovalbumin Y gene product, human plasminogen activator inhibitor 2

- 15 -

and human squamous cell carcinoma antigen, which are similar to the present megakaryocyte differentiation factor in their expected steric structure, and distribution of hydrophobic and hydrophilic amino acids, the N-terminal portion is not cleaved and forms a signal peptide. Accordingly, there is a possibility that in the present megakaryocyte differentiation factor, the N-terminal portion may function as a signal peptide and the megakaryocyte differentiation factor may be secreted without cleavage of the signal peptide. Further, the present megakaryocyte differentiation factor may be modified so that the first methionine is deleted and the second alanine is acetylated.

As hosts, both the prokaryote and eukaryote can be used. As prokaryote, bacteria such as Escherichia coli the genus Bacillus, for example, B. subtilis and the like can be used. As eukaryote, yeast such as the genus Saccharomyces, for example, S. cerevisiae, insect cells such as Spodoptera frugiperda cells, Cabbage looper cells, Bombyx mori cells, animal cells such as human cells, monkey cells, mouse cells and the like can be used. Moreover, insects per se, such as Bombyx mori may be used.

As expression vectors, plasmid, phage, phagemid, virus such as baculo virus, vaccinia virus or the like can be used. A promoter in an expression vector is selected depending on host used. For example, lac promoter, trp promoter and the like can be used as bacterial promoters, and adh1 promoter, pgk promoter and the like can be used as yeast promoters. On the other hand, baculo virus polyhedrin promoter can be used as

- 16 -

insect promoter, and Simian virus 40 early or late promoter can be used for animal cells.

Transformation of a host with an expression vector can be carried out according to conventional procedures well known in the art, and these procedures are described in, for example, Current Protocols in Molecular Biology, John Wiley & Sons. Culturing of a transformant also can be carried out according to a conventional procedure.

Purification of a megakaryocyte differentiation factor from a culture or an insect body can be carried out according to conventional procedures used for isolation and purification of a protein, for example, ultrafiltration, various types of column chromatography such as Q-Sepharose column chromatography and the like.

#### EXAMPLES

Next, the present invention is further explained by Examples.

##### Example 1. Purification of megakaryocyte differentiation factor

###### (1) Culturing of A431 cells

Frozen SBM 330 cells conditioned in a protein-free medium from A431 cells were thawed, and cultured in a primary medium (Ham's F12 medium containing 10% fetal bovine serum). Namely, the cells were plated in 10 T flasks having a culture area of 150 cm<sup>2</sup>, and cultured to confluence at 37°C in the presence of 5% CO<sub>2</sub>. Next, the cells were peeled off with a 0.25% trypsin solution (Chiba Kessei) and subcultured in 10 roller bottles having a culture area of 850 cm<sup>2</sup> at 37°C and 0.5 rpm for about 3 days to recover 1.8 x 10<sup>9</sup> cells. The cultured cells were attached to a ceramic core (S-451) of an Opti-



- 17 -

cell incubator (Charles River Inc. Wilmington, MA) and perfusion culturing was started using 10L of a primary medium.

5 The perfusion culture was carried out at 37°C with supplying oxygen at 150 mmHg. The primary medium was replaced with a protein-free medium as follow. Namely, after culturing for about 7 days in a primary medium, a protein-free medium was supplied to the culture at a rate of 20L/day, and simultaneously the culture  
10 supernatant was recovered from the culture at the same rate. As a result, the primary medium containing serum was substantially completely replaced with the protein-free medium by supplying about 100L of the protein-free medium. Thereafter, cell culture supernatant was  
15 continuously recovered to obtain 1000L of cell culture supernatant. A part (about 300L) of the cell culture supernatant thus obtained was concentrated to 2L using an ultrafiltration membrane (Milipore, Bedford, MA; MW 10,000 cut) and the concentrate was dialyzed against  
20 20 mM Tris/HCl buffer (pH 7.4).

(2) Assay of megakaryocyte differentiation factor using mouse bone marrow cells

Myeloid cells were pushed out of the femur of female BDF<sub>1</sub> mouse and suspended in an  $\alpha$ -MEM medium (Flow  
25 Laboratories, Inc. McLean, VA, USA). Percoll layers having different densities (Pharmacia LKB Biotechnology, Tokyo) were overlaid, and the bone marrow cell suspension was put thereon, followed by centrifugation at 400 x g for 20 minutes. Mononuclear cells collected at  
30 the interface of a layer having a density of 1.07 g/ml and a layer having a density of 1.08 g/ml were recovered

- 18 -

and washed once with  $\alpha$ -MEM containing 10% FBS, and resuspended in the same medium containing 0.5 mM diisopropylfluorophosphate. The suspension was then put into a plastic cell culture dish (Corning, NY, USA) and  
5 cultured at 37°C in 5% carbon dioxide and 95% air for 2 hours. During the culturing, at one hour from the start of culturing, the cell culture dish was replaced with a new one. After the culturing, cells were washed with 10% FBS/ $\alpha$ -MEM three times.

10 The non-adherent bone marrow mononuclear cells thus obtained were suspended in 10% FBS/1% BSA/0.1 mM 2-mercaptoethanol/ $\alpha$ -MEM, and plated in a 96-well microplate (Corning) in an amount of  $5 \times 10^4$  cell/well. If necessary, to a test sample were added 25 U/ml mouse  
15 recombinant IL-3 (Genzyme Corporation, Cambridge, MA, USA) and 1 to 2  $\mu$ g/ml anti-IL-6 antibody (Boehringer Mannheim, Mannheim, FRG). Where anti-IL-6 antibody was added, the test sample and the antibody were preincubated at 37°C for an hour prior to seeding the cells.

20 The culturing was carried out at 37°C in 5% CO<sub>2</sub> - 5% O<sub>2</sub> - 90% N<sub>2</sub> for 4 to 5 days. After culturing cells in each well of the microplate they were washed twice with PBS, and lysed with 180  $\mu$ l of 0.2% (w/v) Triton X-100, 1 mM EDTA, 0.12M NaCl, 50 mM HEPES  
25 (pH 7.5), and 20  $\mu$ l of a substrate, 5.6 mM acetylthiolcholine iodide, was added thereon. After culturing with shaking at a room temperature for an hour, 20  $\mu$ l of the solution was transferred to a microplate for fluorescent assay (Dynatech Micro FLUOR "B" Plate).

30 To the microplate were added 20  $\mu$ l of 0.4 mM

- 19 -

7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarine  
in acetonitrile and 160  $\mu$ l of 0.2% (w/v) Triton X-100,  
1 mM EDTA, 50 mM sodium acetate (pH 5.0), and fluorescent  
emission was measured by a fluorometer (excitation  
5 365 nm, emission 450 nm).

For acetylcholine esterase staining, the cells  
were centrifuged by a Cytospin to adhere the cells onto a  
slide glass, and fixed with 5% glutaraldehyde, 10 mM  
phosphate buffer (pH 6.7) for 15 minutes, and as a  
10 substrate acetylthiocholine was used according to  
Mizoguchi's method (Method for Culturing Hemopoietic Stem  
Cells, Chugai Igaku, 1986, ed. Y. Miura, pp82-88).  
Namely, after fixing the cells, the slide glass was  
washed with 0.1M phosphate buffer (pH 6.0), and to each  
15 slide glass was layered a mixture of 1.5 ml of 0.67 mg/ml  
acetylthiocholine iodide, 0.1M phosphate buffer (pH 6.0),  
0.2 ml of 30 mM  $\text{CuSO}_4$ , 0.2 ml of 5 mM potassium  
ferricyanide and 0.1 ml of 0.1M sodium citrate. The  
slide glass was incubated at a room temperature for  
20 4 hours, and washed with water. May-Gruenwald-Giemsa's  
staining is well known in hematology and was carried out  
using reagents available from E. Merck (Darmstadt, FRG)  
wherein May-Gruenwald's staining was carried out for  
4 minutes and Giemsa's staining was carried out for  
25 10 minutes.

### (3) Purification of megakaryocyte differentiation factor

A culture supernatant concentrate of A431 cells  
was dialyzed and centrifuged to obtain a supernatant  
30 which was then applied to a Matrex Blue A Column  
equilibrated with 20 mM Tris/HCl buffer (pH 7.4), and

after washing the column with the same buffer, a bound fraction was eluted with the same buffer containing 2M NaCl. Megakaryocyte differentiation activity detected by the above-mentioned method was found in the bound

5 fraction. Therefore, the bound fraction was dialyzed against 20 mM Tris/HCl buffer (pH 7.4) and applied to a Q-Sepharose column equilibrated with the same buffer. The column was thoroughly washed and a megakaryocyte differentiation factor was eluted by NaCl gradient (see

10 Fig. 1). This factor was eluted near the position of 0.3 to 0.5M NaCl.

The active fraction obtained from Q-Sepharose was made ammonium sulfate 30% saturation, and was applied to a phenyl Sepharose column equilibrated with 20 mM

15 Tris/HCl buffer (pH 7.4) containing 30% saturation of ammonium sulfate. The megakaryocyte differentiation factor was eluted by simultaneously forming concentration gradients of ammonium sulfate (30% to 0%) and ethyleneglycol (0 to 50%) (see Fig. 2). The

20 megakaryocyte differentiation factor measured in the presence of anti-IL-6 antibody was observed over several fractions in the beginning of the concentration gradient formation.

The fractions thus obtained were combined,

25 thoroughly dialyzed against 50 mM MES/NaOH buffer (pH 6.0), and applied to an S-Sepharose column equilibrated with the same buffer. A bound fraction was eluted by a 0 to 0.5M NaCl concentration gradient (see Fig. 3). Although activity was widely distributed,

30 relatively high activity was found in the beginning of the concentration gradient formation. The fractions

obtained from S-Sepharose were applied to a Hilord 26/60 Super Dex 75 (Pharmacia) column for gel filtration. The column used had be previously equilibrated with the same buffer, and elution was carried out with the same buffer (see Fig. 4). Activity of megakaryocyte differentiation activity was eluted near the position corresponding to a molecular weight of 55 to 57 kDa.

According to the above-mentioned steps, about 80 µg of a fraction showing two bands near 55 to 57 kDa as analyzed by SDS-PAGE was obtained from 300L of a culture supernatant of A431 derived cells (see Fig. 5). The two bands were correlated with the activity (Fig. 1). Accordingly, it was concluded that the two bands observed in said fraction correspond to a desired megakaryocyte differentiation factor.

#### (4) Properties of megakaryocyte differentiation factor

The present megakaryocyte differentiation factor has the following properties.

1) Molecular weight: about 55 kDa (gel filtration and SDS-PAGE) (Figs. 4 and 5)

The present factor exhibits two bands in SDS-PAGE, and there is no difference in mobility between reducing condition and a non-reducing condition. Therefore the factor does not have intermolecular disulfide linkage.

2) Isoelectric point:  $6.5 \pm 0.5$  (Fig. 6)

A several bands are observed in the above-mentioned range.

3) The above-mentioned heterogeneity of the present factor can be explained as heterogeneity in a

- 22 -

sugar chain structure of glycoprotein. Namely, where the present factor is treated with endoglycosidase F which is an asparagine linked sugar removing enzyme, the molecular weight of the present factor decreased to a molecular weight of about 40 kDa, and the heterogeneity also decreased (Fig. 7), in SDS-PAGE. In addition, where a fraction exhibiting a single band and a fraction exhibiting two bands were digested with API and a peptide map was prepared by fractionation by a reversed phase HPLC, the difference was not observed between the two fractions.

4) The present factor contains at least one of the amino acid sequences shown in SEQ ID NO: 1 to 9.

#### 5) Biological activity

Where mouse bone marrow cells were cultured in the presence of a purified megakaryocyte differentiation factor and IL-3, the proliferation and differentiation of megakaryocytes are observed (Figs. 8, 9 and 10). Fig. 8 shows the result of measurement for acetylcholine esterase activity of megakaryocytes; Fig. 9 shows the result of acetylcholine esterase staining of the cultured cells ( $\times 20$ ); and Fig. 10 shows the result of May-Gruenwald-Giemsa's staining of the cultured cells ( $\times 100$ ). In both the Figs. 9 and 10, it is seen that megakaryocytes increased in the presence of a megakaryocyte differentiation factor (B) in comparison with in the absence of the same (A).

#### (5) Structure of megakaryocyte differentiation factor

To characterize the structure of a purified megakaryocyte differentiation factor, the factor

- 23 -

was digested with API and the structures of the resulting fragments were determined. After the digestion of the factor with API, each fragment was recovered by reversed phase HPLC, and the structures for appropriate fractions were determined. As a result, the peptide fragments had amino acid sequences shown in SEQ ID NO: 1 to 9.

Example 2. Determination of Structure of CDNA for megakaryocyte differentiation factor

1. Analysis of cDNA nucleotide sequence of megakaryocyte differentiation factor by PCR (1)

Oligonucleotides NI065 (SEQ ID NO: 10; corresponding to 449-486 of SEQ ID NO: 30) and NI067 (SEQ ID NO: 11; corresponding to 1049-1080 of SEQ ID NO: 30) were synthesized by designing nucleotide sequences on the basis of the amino acid sequences shown in SEQ ID NO: 3 and 4 respectively.

Total RNA was purified from A431 cells using ISOGEN (Wako Pure Chemical) according to the manufacturer's instructions. RNA having poly A was purified from the total RNA, and a reaction was carried out using a 3'-RACE Kit (Gibco BRL). Namely, the above-mentioned oligomer NI065 and oligomer 3'-RACE adaptor primer (SEQ ID NO: 12) attached to the 3'-RACE Kit (Gibco BRL) were used to carry out polymerase chain reaction (PCR) according to instructions included with the kit.

The reaction product was then subjected to second PCR using the primer NI067 and the oligomer 3'-RACE adapter primer included in the 3'-RACE Kit (Gibco BRL) to obtain a DNA fragment of about 900 base pairs. Next, using a direct nucleotide sequence determination method for a PCR product, according to U. Gyllensten et.

- 24 -

al., Proc. Natl. Acad. Sci. USA 85: 7652 (1988), the DNA  
fragment of about 900 base pairs was directly used as a  
reaction substrate to determine a nucleotide sequence of  
a portion representing protein and a portion downstream  
5 of the protein portion using a Taqu Dye Deoxy Terminator  
Cycle Sequencing kit available from Applied Biosystem and  
a fluorescent nucleotide sequencer (Applied Biosystem,  
Type 370A) according to a manufacturers instruction. As  
a result, a sequence from nucleotide number 1081 to 1950  
10 of SEQ ID NO: 30 was shown.

On the basis of this sequence, oligomer KY100  
(SEQ ID NO: 13; corresponding to 1255-1236 of SEQ ID  
NO: 30) was synthesized. The reaction product obtained  
by the PCR using NI065 and the oligomer 3'-RACE adapter  
15 primer attached to the 3'-RACE Kit (Gibco BRL) was used  
as a reaction substrate to carry out a further PCR using  
NI065 and KY100. As a result, a DNA fragment of 807 base  
pairs was obtained.

This DNA fragment of 807 base pairs was  
20 directly used as a reaction substrate to determine its  
nucleotide sequence using Taq Dye Deoxy Terminator Cycle  
Sequencing kit available from Applied Biosystem and a  
fluorescent nucleotide sequencer according to a  
manufactures instruction. As a result, a nucleotide  
25 sequence from nucleotide number 487 to 1080 of SEQ ID  
NO: 30 was shown. On the basis of this sequence,  
oligomers NI073 (SEQ ID NO: 14; corresponding to 864-886  
of SEQ ID NO: 30), NI074 (SEQ ID NO: 15; corresponding to  
1012 to 992 of SEQ ID NO: 30), and NI075 (SEQ ID No: 16;  
30 corresponding to 802-782 of SEQ ID NO: 30) were  
synthesized.



2. Analysis of cDNA nucleotide sequence of  
megakaryocyte differentiation factor by PCR (2)

A. Preparation of mRNA from megakaryocyte  
differentiation factor expressing cell line (A431)

5 From 1.1g of frozen cells of human  
epidermoid carcinoma cell line (A431), 25 µg of mRNA was  
extracted and purified using an RNA extraction kit and an  
mRNA purification kit available from Pharmacia-LKB.

B. Preparation of DNA phage library from  
10 megakaryocyte differentiation factor expressing cell line  
(A431)

(1) Synthesis of cDNA

From 5 µg of the mRNA derived from  
A431, cDNA was synthesized using a cDNA synthesis kit  
15 Time Saver available from Pharmacia-LKB. First 5 µg of  
mRNA dissolved in 20 µl of distilled water treated with  
diethylpyrocarbonate (DEPC) was heated at 65°C for  
10 minutes and cooled on ice. 11 µl of a first strand  
reaction mixture, 1 µl of DTT solution and 1 µl of  
20 130 U/ml NotI/oligomer 18 primer solution (Pharmacia-LKB)  
were added thereto, and the mixture was incubated at 37°C  
for an hour.

The reaction mixture was added to a  
second strand reaction mixture, and the mixture was  
25 incubated at 12°C for 30 minutes and 22°C for an hour,  
and heated at 65°C for 10 minutes. 100 µl of a mixture  
of phenol/chloroform/isoanylalcohol (25:24:1; abbreviated  
as PC hereinafter) was added thereto, and the mixture was  
vigorously stired, centrifuged at 14,000 × 9 for one  
30 minute to obtain a supernatant, which was then

fractionated by a Sephacryl S-400 spin column  
(Pharmacia LKB) to obtain 100 µl of cDNA solution.

(2) Addition of EcoRI adaptor

To 100 µl of the cDNA solution were  
5 added 5 µl of 10 U/ml EcoRI adaptor (Pharmacia LKB),  
30 µl of polyethylene glycol buffer, 1 µl of 1/5 diluted  
ATP solution and 1 µl of T4 DNA ligase, and the mixture  
was incubated at 37°C for an hour. After heating at 65°C  
for 10 minutes, 1.5 µl of ATP solution and 1 µl of T4  
10 polynucleotide kinase were added thereto and the mixture  
was incubated at 37°C for 30 minutes. After heating at  
65°C for 10 minutes, 2 µl of 20 U/µl NotI was added to  
the mixture, which was then incubated at 37°C for an  
hour. 150 µl of PC was added to the mixture, which was  
15 then vigorously stirred and centrifuged at 14,000 × g for  
a minute, and the supernatant was fractionated on  
Sephadex S-400 spin column to obtain 150 µl of cDNA  
solution.

(3) Incorporation of cDNA into phage  
20 vector and in vitro packaging

After digesting with EcoRI and NotI,  
to 15 µl of the cDNA solution were added 2 µg of  
dephosphorylated λgt11D (Pharmacia LKB). After ethanol  
precipitation, the precipitate was dissolved in 8 µl of  
25 ligase buffer solution, and 1 µl of 1/75-diluted ATP  
solution and 1 µl of T4 DNA ligase were added to the  
solution, which was then incubated at 16°C for 30 minutes  
and stored on ice.

An in vitro packaging reaction was

- 27 -

carried out using Gigapack II Gold (Stratagene), and a library of  $3.22 \times 10^6$  pfu of recombinant phages was obtained from said 3 ligase reaction products. Said library was amplified in an E. coli Y1090  $r^-$  host to  
5 obtain  $6.0 \times 10^{10}$  pfu/ml of an A431 phage library stock.

C. Identification and isolation of cDNA fragment for megakaryocyte differentiation factor by PCR

(1) Amplification of cDNA insert fragment in A431 phage library by PCR

10 10  $\mu$ l of a stock solution of  
6.0  $\times 10^{10}$  pfu/ml A431 phage library (corresponding to  
6.0  $\times 10^8$  pfu) was used as a template DNA for PCR  
reaction, and 5  $\mu$ l of 10  $\times$  PCR buffer, 8  $\mu$ l of 1.25 mM  
4 dNTPs, 2  $\mu$ l of 1 OD/ml  $\lambda$ gt11-forward primer ( $\lambda$ gt 11F)  
15 (SEQ ID NO: 17), 2  $\mu$ l of 1 OD/ml  $\lambda$ gt11-reverse primer  
( $\lambda$ gt 11R) (SEQ ID NO: 18), and 1  $\mu$ l of 5 U/ $\mu$ l Taq DNA  
polymerase (Perkin Elmer Cetus) were added, and the total  
volume of the mixture was made to be 50  $\mu$ l with DEPC-  
treated distilled water. 30 reaction cycles of 93°C for  
20 a minute, 55°C for 2 minutes and 72°C for 3 minutes were  
carried out, and the reaction mixture was incubated at  
72°C for 10 minutes. As a result of an analysis by 1%  
Agarose gel electrophoresis, a smeary pattern ranging 0.8  
to 6 kb was shown.

25 (2) PCR analysis using cDNA insert DNA  
amplification fragment mixture as a template and using  
TP7 (SEQ ID NO: 20; corresponding to 683-703 of SEQ ID  
NO: 30), /TP10, TP7/TP6 (SEQ ID NO: 19; corresponding to  
1036-1001 of SEQ ID NO: 30), TP8 (SEQ ID NO: 21;  
30 corresponding to 941-964 of SEQ ID NO: 30), TP10 (SEQ ID

1  $\mu$ l of a 1/5000-diluted solution of  
the above-mentioned PCR reaction product was used as a  
5 template DNA for PCR reaction, and 5  $\mu$ l of 10  $\times$  PCR  
buffer, 8  $\mu$ l of 1.25 mM 4 dNTPs, 2  $\mu$ l each of 1 OD/ml  
primers in combination as described hereinafter and 1  $\mu$ l  
of Perfect Match (Stratagene) were added, and a total  
volume of the mixture was made to 49  $\mu$ l with DEPC-treated  
10 distilled water.

As a result of an analysis of the PCR  
reaction product by 2% agarose gel electrophoresis, bands  
of 354 bp, 354 bp, 96 bp and 96 bp were obtained  
corresponding to primers respectively.

The bands of said PCR amplification products (354 bp) obtained using primers TP7/TP10 and TP7/TP6 were excised from the 2% agarose gel after the electrophoresis, and to the excised agarose gel pieces was added 50 µl of DEPC-treated distilled water, and the





- 31 -

of 2 U/ $\mu$ l E. coli RNase H, and the mixture was incubated at 16°C for 2 hours. 2  $\mu$ l (10 U) of T4 DNA polymerase was added to the reaction mixture, which was then incubated at 16°C for 5 minutes.

5                   The reaction mixture was put on ice, and after adding 10  $\mu$ l of 0.5M EDTA and 150  $\mu$ l of PC thereto, was vigorously stirred and centrifuged at 14,000  $\times$  g for 10 minutes, and 140  $\mu$ l of the supernatant was transferred to a fresh centrifuge tube. 70  $\mu$ l of  
10 7.5M ammonium acetate and 0.5 ml of ethanol were added to the supernatant, which was then allowed to stand at -80°C for 30 minutes. The mixture was centrifuged at 14,000  $\times$  g for 10 minutes, and after removing the supernatant, the precipitate was washed with 0.5 ml of 70% ethanol and  
15 dried under a reduced pressure.

### (3) Addition of BstXI adapter

The above-mentioned cDNA precipitate was dissolved in 25  $\mu$ l of DEPC-treated distilled water, 10  $\mu$ l of 5  $\times$  T4 DNA ligase buffer, 10  $\mu$ l of BstXI adapter  
20 (Invitrogen) and 5  $\mu$ l of T4 DNA ligase were added to the solution, which were then incubated at 16°C for 16 hours. 50  $\mu$ l of PC was added to the mixture, which was the vigorously stirred, and centrifuged at 14,000  $\times$  g for 5 minutes. 45  $\mu$ l of the supernatant was transferred to a  
25 fresh centrifuge tube. 25  $\mu$ l of 7.5M ammonium acetate and 150  $\mu$ l of ethanol were added to the tube, which was stirred and allowed to stand at -80°C for 30 minutes. After centrifuging at 14,000  $\times$  g for 10 minutes to remove supernatant, the precipitate was washed with 0.5 ml of

70% ethanol and dried under a reduced pressure.

(4) NotI digestion

The above-mentioned cDNA precipitate was dissolved in 41 µl of DEPC-treated distilled water, and 5 µl of REAct 7 buffer and 4 µl of NotI were added to the solution, which was incubated at 37°C for 2 hours. 50 µl of PC was added to the mixture, which was then vigorously stirred and centrifuged at 14,000 × g for 10 minutes, and 45 µl of the supernatant was transferred into a centrifuge tube.

(5) Elimination of adapter and size fractionation of partial cDNA

The above-mentioned cDNA solution was fractionated using a Quick Spin Column Linker 5 (Boehringer Mannheim). 50 µl of 40 µg/µl cDNA was obtained.

(6) Incorporation of cDNA into phagevector and transformation of E. coli

To 37.5 µl of the above-mentioned cDNA solution were added 12.5 µl of pCR/CMV (Invitrogen) vector (29 µg/µl) digested with Not I and BstXI, and further added were 400 µl of Takara Ligation kit A solution and 50 µl of B solution, and the mixture was incubated at 16°C for 30 minutes, and 1 ml of Max Efficiency DH5α competent cells (BRL) were transformed to obtain 71,550 recombinant clones. All colonies were collected from plate ( $2.86 \times 10^7$  cells/ml) and stored at -80°C in the presence of 20% glycerol.

B. Screening of megakaryocyte differentiation factor cDNA by colony hybridization



Using the cDNA plasmid library derived from A431 cell line, a total of 227,000 (3700/plate) colonies were formed on 60 plates of 9 cm diameter, and the colonies were replicated to nitrocellulose filters.

5 A probe was prepared by carrying out PCR (as described hereinbefore) using primer NI 067 and 3'-RACE adaptor (GIBCO BRL) to obtain a 900 bp PCR product, digesting the PCR product with Bam HI to obtain two DNA fragments (0.5 kb and 0.4 kb) and nick-translating the DNA

10 fragments with [ $\alpha$ -<sup>32</sup>P]dCTP.

For the colony hybridization, the filter was incubated in 5 × SSC, 25 mM phosphate buffer (pH 7.4), 5 × Denhardt's solution, 1% SDS, 100 µg/ml heat denatured salmon sperm DNA and 50% formamide at 42°C for

15 18 hours, and washed with 5 × SSC, 0.1% SDS at 40°C for 20 minutes and 45°C for 20 minutes. Detection was carried out by exposing BAS 2000 (Fuji Film) for 18 hours.

First, second and third screening was carried out to obtain 4 clones, i.e., TP290, TP308, TP310 and TP317. The length of insert cDNA was 1.2 kb, 1.1 kb, 1.2 kb and 1.2 kb respectively. The TP290, TP310 and TP317 cover a region downstream from the nucleotide number 685 of SEQ ID NO: 30.

25 4. Analysis of cDNA nucleotide sequence coding for megakaryocyte differentiation factor by PCR (3)

A. Preparation of mRNA from megakaryocyte differentiation factor expressing cell line (HPC-Y11)

From 1.1g of frozen cells of human

30 pancreatic cancer cell line (HPC-Y11), 50 µg of mRNA was extracted and purified using an RNA extraction kit and

mRNA purification kit available from Pharmacia-LKB.

B. Preparation of cDNA phage library from megakaryocyte differentiation factor expressing cell line (HPC-Y11)

5 (1) Synthesis of cDNA

From 5 µg of mRNA derived from HPC-Y11, cDNA was synthesized using a Pharmacia-LKB's Time Saver cDNA synthesis kit. First, 5 µg of mRNA was dissolved in 20 µl of diethylpyrocarbonate (DEPC)-treated distilled water, and the solution was heated at 65°C for 10 minutes and cooled on ice. 11 µl of first strand reaction mixture, 1 µl of DTT solution and 1 µl of NotI/oligomer 18 primer solution were added to the mixture, which was then incubated at 37°C for an hour.

15 100 µl of second strand reaction mixture was added to the mixture, which was then incubated at 12°C for 30 minutes and at 22°C for an hour, and heated at 65°C for 10 minutes. 100 µl of phenol-chloroform-isoamyl alcohol (25:24:1, abbreviated as PC) was added to the mixture, which was then vigorously stirred and centrifuged at 14,000 × g for a minute, and the supernatant was fractionated using a Sephacryl S-400 spin column (Pharmacia-LKB) to obtain 100 µl of cDNA solution.

25 (2) Addition of EcoRI adaptor

To 100 µl of the cDNA solution, were added 5 µl of EcoRI adaptor (Pharmacia-LKB), 30 µl of polyethylene glycol, 1 µl of ATP solution and 1 µl of T4 DNA ligase, and the mixture was incubated at 37°C for an hour. After heating at 65°C for 10 minutes, 1.5 µl of

30

ATP solution and 1  $\mu$ l of T4 polynucleotide kinase were added to the mixture, which was then incubated at 37°C for 30 minutes. After heating at 65°C for 10 minutes, 12  $\mu$ l of Not I was added to the mixture, which was then incubated at 37°C for an hour. 150  $\mu$ l of PC was added to the mixture, which was then vigorously stirred and centrifuged at 14,000  $\times$  g for a minute, and the supernatant was fractionated using a Sephacryl S-400 spin column to obtain 150  $\mu$ l of cDNA solution.

(3) Incorporation of cDNA into phage vector and in vitro packaging

To 15  $\mu$ l of the cDNA solution, was added 2  $\mu$ g of  $\lambda$ gt 11D (Pharmacia-LKB) which had been digested with EcoRI and Not I and dephosphorylated, and after ethanol precipitation, the precipitate was dissolved in 8  $\mu$ l of ligase buffer. 1  $\mu$ l of 1/75-diluted ATP solution and 1  $\mu$ l of T4 DNA ligase were added to the mixture, which was then incubated at 16°C for 30 minutes and stored on ice. An in vitro packaging reaction was carried out using a Giga Pack II Gold (Stratagene), and 5.34  $\times$  10<sup>6</sup> pfu of recombinant phages were obtained from the above-mentioned 3 ligase reaction products. The library was amplified in E. coli Y1090 r<sup>-1</sup> host to obtain a stock of 1.7  $\times$  10<sup>11</sup> pfu/ml HPC-Y11 phage library.

C. Identification and isolation of 5'-portion of megakaryocyte differentiation factor cDNA

(1) Amplification by PCR of HPC-Y11 phage library cDNA insert DNA fragment primer NI074 upstream portion.

To 1  $\mu$ l (corresponding to

1.7  $\times 10^9$  pfu) of 6.0  $\times 10^{10}$  pfu/ml HPC-Y11 phage library stock solution as a template DNA for PCR reaction, were added 5  $\mu$ l of 10  $\times$  PCR buffer, 8  $\mu$ l of 1.25 mM 4 dNTPs, 1  $\mu$ l of 10 OD/ml  $\lambda$ gt11-forward F1 primer (SEQ ID NO: 25),  
5 1  $\mu$ l of 50 D/ml NI074 primer and 1  $\mu$ l of Perfect Match (Stratagene), and a total volume was made to be 49  $\mu$ l with DEPC-treated distilled water.

After heating the reaction mixture at 95°C for 5 minutes and 60°C for 5 minutes, 1  $\mu$ l of 5 U/ $\mu$ l  
10 Taq DNA polymerase (Perkin Elmer Cetus) was added thereto, and 35 reaction cycles of 94°C for a minute, 60°C for a minute and 72°C for 2 minutes were carried out, followed by incubation at 72°C for 10 minutes. A  
result of analysis by 2% agarose gel electrophoresis  
15 showed a smeary pattern ranging from 0.3 to 6 kb.

(2) PCR analysis using a template which is a PCR amplification fragment mixture prepared by using  $\lambda$ gt11F1/NI074 primer, and using as primers  $\lambda$ gt11F2 (SEQ ID NO: 26)/NI075,  $\lambda$ gt11F2/TP12 (SEQ ID NO: 28;  
20 corresponding to 703-683 of SEQ ID NO: 30),  $\lambda$ gt11F2/TP11 (SEQ ID NO: 27; corresponding to 619-599 of SEQ ID NO: 30),  $\lambda$ gt11F2/TP13 (SEQ ID NO: 29; corresponding to 595-575 of SEQ ID NO: 30), TP7/NI074, TP7/NI075, and NI073/NI074

25 1  $\mu$ l of a 1/100 diluted solution of the above-mentioned PCR reaction product was used for a template DNA for PCR reaction, and 5  $\mu$ l of 10  $\times$  PCR buffer, 8  $\mu$ l of 1.25 mM 4 dNTPs, 0.5  $\mu$ l each of 10 OD/ml primers in combination as described hereinafter, and 1  $\mu$ l  
30 of Perfect Match (Stratagene) were added thereto, and a

total of the reaction mixture was made to be 49  $\mu$ l with DEPC-treated distilled water. The reaction mixture was heated at 95°C for 5 minutes and at 60°C for 5 minutes, 1  $\mu$ l of 5 U/ $\mu$ l Taq DNA polymerase (Perkin Elmer Cetus) was added thereto, and 35 reaction cycles of 94°C for a minute, 60°C for 2 minutes and 72°C for 2 minutes were carried out, followed by an incubation at 72°C for 10 minutes.

As primers,  $\lambda$ gt11F2/NI075,  $\lambda$ gt11F2/TP12,  $\lambda$ gt11F2/TP11,  $\lambda$ gt11F2/TP13, TP7/NI074, TP7/NI075, and NI073/NI074 were used. As a result of 2% agarose gel electrophoresis, bands of 969 bp, 870 bp, 786 bp, 762 bp, 330 bp, 120 bp and 149 bp were obtained corresponding to the primers.

(3) PCR analysis and primary sequence analysis using  $\lambda$ gt11F/TP11 and  $\lambda$ gt11F/TP13 primer, for PCR amplification product (969 bp) prepared using F2/NI075 primers

0.5  $\mu$ l of the above mentioned PCR reaction product (969 bp) prepared by using  $\lambda$ gt11F2/NI075 as primers was used as a template DNA for PCR reaction, and 5  $\mu$ l of 10  $\times$  PCR buffer, 8  $\mu$ l of 1.25 mM 4 dNTPs, 1  $\mu$ l of 10 OD/ml  $\lambda$ gt11F primer, 1  $\mu$ l of 10 OD/ml TP11 primers or 1  $\mu$ l of 10 OD/ml TP13 primers, and 1  $\mu$ l of Perfect Match (Stratagene) were added, and the total volume was made to be 49  $\mu$ l with DEPC-treated distilled water.

The reaction mixture was heated at 95°C for 5 minutes and 60°C for 5 minutes, 1  $\mu$ l of 5 U/ $\mu$ l Taq DNA polymerase (Perkin Elmer Setus) was added

- 38 -

thereto, and 35 reaction cycles of 94°C for a minute, 60°C for 2 minutes and 72°C for 2 minutes were carried out, followed by an incubation at 72°C for 10 minutes.

Bands of PCR reaction products

5 (678 bp and 654 bp, respectively) were excised from 2% agarose gel after electrophoresis, extracted and purified, and inserted into pCRII (Invitrogen), which was then used to transform E. coli IN VαF' (Introgen). Plasmid DNA was extracted and purified from the  
10 transformant, and digested with EcoRI to confirm that a 0.7 kb DNA fragment was inserted. The primary sequence of the inserted DNA fragment was analyzed by using M13 forward primer M13F and M13 reverse primer M13R (Applied Biosystems automated sequencer Model 370A).

15 As a result, a sequence of 619 bp corresponding to nucleotide number 1 to 619 of SEQ ID NO: 30 was found, and the nucleotide sequence of 133 nucleotides consisting of nucleotide number 487 to 619 of SEQ ID NO: 30 conformed to the N-terminus of the primary  
20 sequence found in Example 2.1. In this 619 bp sequence there are 19 amino acids of SEQ ID NO: 3 (VERVDFTNHLEDTR RNINK from DNA nucleotide sequence) and 5 amino acids (LYDAK) of SEQ ID NO: 7, and it was clarified that this PCR reaction products (each 0.7 kb) was a part of cDNA  
25 coding for megakaryocyte differentiation factor.

It was considered that the translation starting methionine corresponds to 74th nucleotide, and 5'-non translational region consisted of 73 bp. Accordingly, it was clarified that these PCR  
30 reaction products (each 0.7 kb) contain N-terminus of structural gene for megakaryocyte differentiation factor.

- 39 -

5. Analysis of cDNA nucleotide sequence coding for megakaryocyte differentiations factor by PCR (4)

Among an N-terminal portion of structural gene for a megakaryocyte differentiation factor derived from HPC-Y11 and a sequence considered to be a 5'-non-translational region, obtained in the section C(3), the sequence of nucleotide numbers 12 to 31 of SEQ ID NO: 30 which is a sequence part considered to be a 5'-non-translational region was used as a basis to synthesize an oligomer NI 083 (SEQ ID NO: 31).

The RNA having poly A prepared from A431 cells in the section 1., a Preamplification System (Gibco BRL) and random hexamers attached to the System were used to synthesize first strand cDNA according to included instructions, and PCR was carried out by using NI083 and NI074 as well as Ampli Taq (Takara). As a result, a DNA fragment of 1001 bp which is a cDNA fragment for a megakaryocyte differentiation factor was obtained.

This DNA fragment, a PCR product, of 1001 bp was directly used as a substrate for sequencing on a Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystem) and a fluorescent sequencer (Applied Biosystem Type 370A) according to the included manufacturers instruction. As a result, a sequence of nucleotide numbers 32-486 of SEQ ID NO: 30 was found. In addition, a result obtained for nucleotide numbers 487 to 991 of SEQ ID NO: 30 conformed to the sequence obtained in the section 1.

By combining with the sequence obtained in the section 1., the nucleotide sequence 32-1950 of SEQ ID NO: 30 which is cDNA nucleotide sequence coding for a

- 40 -

megakaryocyte differentiation factor of A431 cell was determined.

For this nucleotide sequence, all of possible 3 reading frames were mechanically translated into amino acid sequences and it was found that one of them has a region which can be translated to a continuing amino acid sequence containing all the amino acid sequences shown in SEQ ID NO: 1 to 9, and the reading frame of the megakaryocyte differentiation factor was determined.

It was found that in this reading frame a codon for methionine (nucleotide numbers 74 to 76) found at a translation start position exists, and from this position a region which can be translated to an amino acid sequence containing the amino acid sequences shown in SEQ ID NO: 1 to 9 continues up to the nucleotide position 1213 of SEQ ID NO: 30, and it was determined that the nucleotide sequence from the position 74 to the position 1213 of SEQ ID NO: 30 is the region translated to megakaryocyte differentiation factor.

The nucleotide sequence GCAATGG (nucleotide numbers 71 to 77 of SEQ ID NO: 30) encompassing the methionine codon of nucleotide numbers 74 to 76 of SEQ ID NO: 30 corresponds to a sequence (G/A-N-N-A-T-G-G) encompassing a methionine codon frequently present at a translation start site found by M. Kozak, Nucleic Acids Research (1981) Vol. 9, p5233-5252.

Primary sequence of megakaryocyte differentiation factor was thus clarified, the number of amino acids was 380 in the structural gene, an expected molecular weight was 42904.43, and an expected isoelectric point was 6.79. SEQ ID NO: 1 corresponds to



- 41 -

amino acid numbers 188 to 196 of SEQ ID NO: 30; SEQ ID  
NO: 2 corresponds to amino acid numbers 181 to 187 of SEQ  
ID NO: 30; SEQ ID NO: 3 corresponds to the amino acid  
numbers 126 to 144 of SEQ ID NO: 30; SEQ ID NO: 4  
5 corresponds to the amino acid numbers 325 to 341 of SEQ  
ID NO: 30; SEQ ID NO: 5 corresponds to the amino acid  
numbers 289 to 297 of SEQ ID NO: 30; SEQ ID NO: 6  
corresponds to the amino acid numbers 305 to 324 of SEQ  
ID NO: 30; SEQ ID NO: 7 corresponds to the amino acid  
10 numbers 121 to 125 of SEQ ID NO: 30; and SEQ ID NO: 8  
corresponds to the amino acid numbers 284 to 288 of SEQ  
ID NO: 30; and SEQ ID NO: 9 corresponds to the amino acid  
numbers 204 to 213 of SEQ ID NO: 30. In addition, A poly  
A addition signal AATAAA sequence is present at  
15 nucleotide numbers 1933 to 1998 of SEQ ID NO: 30.

Example 3. Isolation and identification of cDNA  
coding for megakaryocyte differentiation factor from A431  
by PCR and construction of expression vector

Oligomers NI078 (SEQ ID NO: 32) and NI079 (SEQ ID  
20 NO: 33) were synthesized on the basis of the sequence  
(SEQ ID NO: 30) obtained in Example 2. Note in the  
NI078, the sequence of nucleotide numbers 13 to 37  
encompassing the translation start methionine codon  
conforms to the sequence of the nucleotide numbers 74 to  
25 98 of SEQ ID NO: 30 and an EcoRI recognizing site  
(nucleotide numbers 4 to 9) and an Nru I recognizing site  
(nucleotide numbers 8 to 13) were artificially added; and  
in the NI079, the sequence of the nucleotide numbers 17  
to 49 conforms to the nucleotide sequence of the  
30 nucleotide numbers 1237 to 1269 of SEQ ID NO: 30, and an  
EcoRI recognizing site (nucleotide numbers 3 to 8) and an

- 42 -

Not I recognizing site (nucleotide numbers 9 to 16) were artificially added.

5 The RNA having poly A prepared from A431 cells in Example 2 section 1., a Preamplification System (Gibco BRL), and Oligomers, i.e., random hexamers included in the system were used according to the included manufacturer's instructions to synthesize first strand cDNA, and PCR was carried out using the synthesized DNA as a template and NI078 and NI079 as primers and using  
10 Ampli Taq (Perkin Elmer Cetus). As a result, a DNA fragment of 1224 base pairs which is a cDNA fragment for a megakaryocyte differentiation factor and has all information relating to megakaryocyte differentiation factor, was obtained.

15 This DNA fragment was treated with EcoRI to generate EcoRI cohesive sites at both the ends of the cDNA coding for a megakaryocyte differentiation factor in virtue of EcoRI recognizing sites artificially added to the oligomers NI078 (SEQ ID NO: 32) and NI079 (SEQ ID  
20 NO: 33). This cDNA fragment coding for megakaryocyte differentiation factor was introduced into a mammalian expression vector pdKCR-DHFD at its EcoRI recognizing site to obtain pdKCR-DHFR-TPO55.

The animal cell expression vector pdKCR-dhfr  
25 (Oikawa, S. et. al., Biochem. Biophys. Res. Commun. 164, 39, 1989) is a derivative of pKCR (O' Hare et. al., Pro. Natl. Acad. Sci. USA, 78, 1527, 1981) and has SU 40 early promoter and a rabbit  $\beta$ -globin gene and dhfr (dehydrofolate reductase) gene. Note, a host transformed  
30 with the expression vector, was designated as Escherichia coli SBM 308, and deposited with the Fermentation

- 43 -

Research Institute, Agency of Industrial Science and  
Technology, 1-3, Higashi 3-chome, Tsukuba-shi, Ibaraki,  
Japan as FERM P-11506 on June 7, 1990, and transferred to  
an international deposition under the Budapest treaty as  
5 FERM BP-4197 on February 18, 1993.

The clone pdKCR-DHFR-TP055 containing megakaryocyte  
differentiation factor cDNA which was incorporated in to  
pdKCR-DHFR was sequenced using a Taq Dye Deoxy Terminator  
Cycle Sequencing kit (Applied Biosystem) and a  
10 fluorescent sequencer (Applied Biosystem Type 370A)  
according to included instructions. As a result, the  
determined nucleotide sequence conformed to the sequence  
of the nucleotide numbers 99 to 1236 of SEQ ID NO: 30 and  
oligomers NI078 and NI079. In addition, it was confirmed  
15 by the sequencing that a megakaryocyte differentiation  
factor cDNA inserted into the vector is in correct  
orientation in relation to an expression vector promoter.

As shown in the above, once the information of SEQ  
ID NO: 30 is provided, it is easy for a person skilled in  
20 the art that the nucleotide sequence is determined by  
amplifying cDNA coding for megakaryocyte differentiation  
factor in total or in a optional portion on megakaryocyte  
differentiation factor expressing cell line (for example,  
A431) and bonded in a optional expression vector.

25 Example 4. Expression of megakaryocyte  
differentiation factor in Bombyx mori

(1) Construction of Bombyx mori expression vector

A megakaryocyte differentiation factor cDNA  
clone pdKCR-DHFR-TP055 was digested with NotI to cleave  
30 the NotI recognizing site artificially added to the  
NI079. The NotI cohesive end thus generated was blunt-

- 44 -

ended using a blunting kit available from Takara Shuzo,  
and to the blunt end was added an XbaI linker (Takara  
Shuzo) according to an attached instruction. The plasmid  
thus obtained was digested simultaneously with NruI and  
5 XbaI to cleave the NruI recognizing site artificially  
added to the NI078 and the XbaI recognizing site of the  
XbaI linker introduced to prepare a megakaryocyte  
differentiation factor cDNA fragment having an NruI  
cohesive end and an XbaI cohesive end. This DNA fragment  
10 was inserted at the Nru I recognizing site into a  
baculovirus transfer vector for Bombyx mori nuclear  
polyhedrosis virus, pBm4 (available from Department of  
Insect Genetics and Bleeding National Institute of  
Sericultural and Entomological Science, Ohwashi, Tukuba,  
15 Ibaraki 305, Japan) simultaneously digested with NruI and  
XbaI to obtain pBm4-TP055.

(2) Construction of TP055 recombinant virus

A cell line derived from Bombyx mori embryonic,  
BoMo15AIIc (available from Department of Insect Genetics  
20 and Bleeding National Institute of Sericultural and  
Entomological Science, Ohwashi, Tukuba, Ibaraki 305,  
Japan) was subcultured in a medium containing 10% fetal  
bovine serum (FBS: GIBCO BRL) and 500 µg/ml gentamicin  
in MGM 448 at 25°C. TP055 recombinant virus was  
25 constructed by co-introducing Bombyx mori nuclear  
polyhedrosis virus gene DNA and pBm4-TP055 plasmid DNA  
into Bombyx mori cultured cells by, for example, calcium  
phosphate co-precipitation method.

Namely, 2 µg of genomic DNA of wild type virus  
30 B6E (available from Department of Insect Genetics and  
Bleeding National Institute of Sericultural and

- 45 -

Entomological Science, Ohwashi, Tukuba, Ibaraki 305,  
Japan) and 10 µg of the transfer plasmid pBm4-TP055 were  
dissolved in 240 µl of sterile purified water, and to the  
solution was added the same volume of 0.5 M CaCl<sub>2</sub> and  
5 0.1M HEPES, and the mixture was mixed and allowed to  
stand at a room temperature for 10 minutes. To the  
mixture was added 480 µl of 0.2M NaCl, 0.05M HEPES,  
0.75 mM NaH<sub>2</sub>PO<sub>4</sub> and 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, and the mixture was  
stirred for a few second and allowed to stand at a room  
10 temperature for 20 to 30 minutes to form calcium  
phosphate gel containing the genomic DNA and the plasmid.

Next, 960 µl of calcium phosphate gel  
suspension containing the viral genomic DNA and the  
transfer vector was added to 4 ml of BoMo15AIIc cells in  
15 a 25 cm<sup>2</sup> T flask (T25, Corning), and the mixture was  
allowed to stand for 12 hours. The medium was replaced  
with a fresh MGM448 (containing 10% FBS and the  
antibiotics), and culturing was carried out for 25°C. On  
the sixth day the cultured medium was recovered as a  
20 viral solution.

The cultured medium was centrifuged to obtain a  
clear supernatant, which was then diluted, and added to  
BoMo15AIIc cells cultured on the microtiter plate, and  
after 8 days a culturing medium in which viral infection  
25 was microscopically observed but a polyhedral body was  
not formed, was selected (by a limited dilution method).  
The cultured medium was recovered. Contamination with a  
wild virus in the viral solution factor was not observed.

A recombinant virus thus constructed,  
30 containing a DNA coding for megakaryocyte differentiation  
factor was designated as TPO55-BmNPV.



- 47 -

extracted from TPO55-BmNPV-infected cells, and it was shown that the mRNA was expressed in the TPO55-BmNPV-infected cells. On the other hand expression of mRNA which hybridized with the probe DNA was not observed in the B6E-infected cells and un-infected cells.

(4) Preparation of solution of recombinant virus

About  $1 \times 10^6$  BoMol5AIIc cells were cultured in 4 ml of MGM448 containing 10% FBS on the bottom of a 25 cm<sup>2</sup> flask for 2 days, and to this culture was added 10 µl of the culture medium of BoMol5AIIc cells containing the recombinant virus cloned in the above section (2). After culturing at 25°C for 14 days, the culture medium was centrifuged at 1000 rpm for 5 minutes to obtain a supernatant as a recombinant virus solution.

(5) Preparation of hemolymph of *Bombyx mori*

50 µl/head of a viral solution of the  $10^{-1}$ -diluted recombinant virus solution obtained in the above section (3) or a  $10^{-1}$ -diluted wild type virus B6E solution was injected to *Bombyx mori* larvae at 5th instar, and the silkworms were fed with commercially available artificial feed (Morus; Katakura Kogyo) at 20°C for 4 to 5 days. The abdomens of 50 silkworms were cut, and an extract containing the hemolymph and the content in the central intestine was taken in a plastic tube cooled with ice, and centrifuged to obtain a supernatant.

(6) Confirmation of activity of megakaryocyte differentiation factor

50 ml of the hemolymph of the silkworms obtained in the above section (5) was thoroughly dialyzed against a 20 mM Tris/HCl (pH 7.4) buffer, and applied to a Matrex Blue A column ( $\phi 2.5 \times 15$  cm) equilibrated with

- 48 -

the same buffer. The column was thoroughly washed with the same buffer to eliminate a unbound fraction, and a bound protein was eluted by a concentration gradient of 0 to 1M NaCl. An elution profile for megakaryocyte  
5 differentiation activity of the hemolymph obtained from silkworms injected with the recombinant virus was compared with that for a wild type virus.

As seen from Fig. 12, megakaryocyte differentiation activity in the hemolymph from silkworms  
10 injected with the recombinant virus was significantly higher than that for the wild type virus.

Although Bombyx mori vacuovirus transfer vector pBm4, Bombyx mori nuclear polyhedrosis virus PbE and Bombyx mori cells BoMo15AIIc were used in Example 4,  
15 the present invention is not limited to the use of these materials. Namely, other baculovirus transfer vector (such as pBK283, pBKblue, available from Funakoshi) Bombyx mori nuclear polyhedrosis virus (such as purified DNA available from Funakoshi), Bombyx mori cells (such as  
20 BmN4 cells, available from Funakoshi) can be easily used by a person with ordinary skill in the art to obtain a megakaryocyte differentiation factor.

A megakaryocyte differentiation factor of the present invention accelerates formation of megakaryocytes  
25 from myeloid cells in the presence of IL-3. The present megakaryocyte differentiation factor plays an important role in differentiation of megakaryocytes and acts in vivo as a thrombopoietin. Accordingly, the present megakaryocyte differentiation factor may be medicaments  
30 effective to not only various diseases involving decrease of platelets but also for control of the number of



- 49 -

platelets decreasing by radiation in the case of bone marrow transradiation, or for control of the number of platelets in chemotherapy of cancers.